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REVIEW

Identifying circulating microRNAs as biomarkers of cardiovascular disease: a systematic review

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Abstract

The aim of the present study is to identify microRNAs (miRs) with high potential to be used as biomarkers in plasma and/or serum to clinically diagnose, or provide accurate prognosis for survival in, patients with atherosclerosis, coronary artery disease, and acute coronary syndrome (ACS). A systematic search of published original research yielded a total of 72 studies. After review of the risk of bias of the published studies, according to Cochrane Collaboration and the QUADUAS Group standards, 19 studies were selected. Overall 52 different miRs were reported. In particular, miR-133a/b (5 studies), miR-208a/b (6 studies), and miR-499 (7 studies) were well studied and found to be significant diagnostic and/or prognostic markers across different cardiovascular disease progression stages. miR-1 and miR-145b are potential biomarkers of ACS; miR-1 with higher sensitivity for all acute myocardial infarction (AMI), and miR-145 for STEMI and worse outcome of AMI. But when miRs were studied across different ACS study populations, patients had varying degrees of coronary stenosis, which was identified as an important confounder that limited the ability to quantitatively pool the study results. The identified miRs were found to regulate endothelial function and angiogenesis (miR-1, miR-133), vascular smooth muscle cell differentiation (miR-133, miR-145), communication between vascular smooth muscle and endothelial cell to stabilize plaques (miR-145), apoptosis (miR-1, miR-133, miR-499), cardiac myocyte differentiation (miR-1, miR-133, miR-145, miR-208, miR-499), and to repress cardiac hypertrophy (miR-133). Their role in these processes may be explained by regulation of shared RNA targets such as cyclin-dependent kinase inhibitor 1A (or p21), ETS proto-oncogene 1, fascin actin-bundling protein 1, hyperpolarization-activated cyclic nucleotide-gated potassium channel 4, insulin-like growth factor 1 receptor LIM and SH3 protein 1, purine nucleoside phosphorylase, and transgelin 2. These mechanistic data further support the clinical relevance of the identified miRs. miR-1, miR-133a/b, miR-145, miR-208a/b, and miR-499(a) in plasma and/or serum show some potential for diagnosis of cardiovascular disease. However, biased selection of miRs in most studies and unexplained contrasting results are major limitations of current miR research. Inconsistencies need to be addressed in order to definitively identify clinically useful miRs. Therefore, this paper presents important aspects to improve future miR research, including unbiased selection of miRs, standardization/normalization of reference miRs, adjustment for patient comorbidities and medication, and robust protocols of data-sharing plans that could prevent selective publication and selective reporting of miR research outcomes.

Keywords Circulating microRNA • Biomarkers • Coronary artery disease • (Disease) Progression • Acute coronary syndrome

1. Introduction

MicroRNAs (miRs) are endogenous, non-coding, and small (18–22 nucleotides) RNA molecules. miRs are recruited to the RNA-induced silencing complex (RISC) and regulate the output of protein-coding genes through diverse mechanisms.[1] The interaction of miRs with the 3′ untranslated region (3′ UTR) of protein-coding genes is considered as the main mechanism, which usually leads to a decrease in protein

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output either by mRNA degradation or by translational repression.[2] Recent studies have also suggested that miRs can interact with the 5′ UTR of protein-coding genes via complementarity and cause translational repression[3] or activation of the targeted proteins.[4] Similarly, miRs can also target the coding sequence and repress the translation of targeted genes.[5] Moreover, some miRs can interact with regulatory protein complexes, such as argonaute 2 and fragile X mental retardation-related protein 1, and indirectly up-regulate the translation of a target gene.[6] miRs are produced by all cell types, and the same miR may be derived from a variety of cell sources, such as endothelial cells, monocytes and macrophages, vascular smooth cells, and platelets and eventually are secreted in blood. There, degradation may be circumvented by being packaged in microparticles (exosomes, microvesicles, and apoptotic bodies) or bound with proteins or high-density lipoproteins (HDLs).[7–9] In this publication, all miRs detected in plasma or serum are reviewed, independent of their cell origin or their package in microparticles and/or lipoproteins.

Several miRs were shown to take part in the pathogenesis of coronary artery disease (CAD)[10,11] and atherosclerosis.[12–14] Therefore, we aim to determine whether miRs in plasma and/or serum differentiate between stable and unstable CAD (or acute coronary syndromes; ACSs), unstable angina (UA) or acute myocardial infarction (AMI) within ACS, STEMI, or non-STEMI AMI, or are markers of worse outcome such as (cardiac) death. In preparing this review, the authors followed current guidance on producing high-quality systematic reviews and meta-analyses of diagnostic test accuracy, by both the Cochrane Collaboration[15] and the QUADUAS Group.[16] As per the Cochrane Collaboration,[17] when studies that lack methodological rigor or have a high risk of bias are combined in a meta-analysis, the uninformative or erroneous results can lead to wasting further resources on future research or can even lead to harm if patients are wrongly diagnosed. The authors also followed the PRISMA[18] reporting guidelines for systematic reviews (see Supplementary material online, *File S1*). In following these standards, we aimed at providing quality evidence for the rational choice of miRs to focus on in future research, identifying the miRs with high potential for clinical applicability to distinguish patients with the disease from those without. In the second and third parts of this review, we searched for and report on mechanistic data on the clinical relevance and on the target genes of miRs with high potential identified in the first part of our study.

2. Methods

2.1 Information resources, search, and study selection

The strategy and outcome of the literature search are illustrated in *Figure 1*.

We searched the PubMed database for English language articles related to miRs as biomarkers in atherosclerosis, CAD, and ACS in humans, with the last search taking place on 7 October 2015. No lower date limit was used. The search strategy was performed by two independent researchers (D.G. and R.N.), encompassing a MESH search: (*'Coronary Artery Disease'*[*Mesh*] *OR 'Atherosclerosis'*[*Mesh*] *OR 'Myocardial Infarction'*[*Mesh*] *OR 'Acute Coronary Syndrome'*[*Mesh*]) AND (*'MicroRNAs'*[*Mesh*]) AND (*Humans*[*Mesh*] *and English*[*lang*]), identifying 412 titles. As well, a second more extended search of typical keywords was performed using: ((*mir OR MicroRNA*) NOT (*mir*[*author*])) AND (*atherosclerosis OR CAD OR ACS OR myocardial infarction*) and (*plasma OR serum OR blood OR circulating OR circulation*) AND (*Humans*[*Mesh*] *and English*[*lang*]) NOT (*Review*[*Publication Type*]). This text search resulted in the identification of 259 articles: with 201 articles being duplicates of the MESH search strategy and 58 new unique articles. In addition, 17 articles were identified from reference lists and author archives. From the 487 unique articles, a title screen was performed by three authors (R.N., D.G., and P.H.), and articles were excluded based on the following criteria:

- Experiments in molecular biology/biochemistry ($n = 75$),
- Experiments in pharmacology ($n = 23$),
- miR examined in cells or tissues other than blood ($n = 30$),
- Medical field other than cardiology ($n = 56$),
- Topic out of scope (though cardiology) ($n = 84$),
- Experiments with laboratory animals ($n = 14$),
- Other experiments in genetics ($n = 23$),
- Article is a review, editorial, comment, or interview, not an original research article ($n = 64$),
- Target population out of scope ($n = 3$).

Title screening left a total of 115 papers, which were then screened by abstract. The exclusion criteria were as follows:

- Experiments in molecular biology/biochemistry ($n = 4$),
- Experiments in pharmacology ($n = 1$),
- miR examined in cells or tissues other than blood ($n = 4$),
- Medical field other than cardiology ($n = 1$),
- Topic out of scope (though cardiology) ($n = 3$),
- Experiments with laboratory animals ($n = 1$),
- Other experiments in genetics ($n = 4$),
- Article is a review, editorial, comment, or interview, not an original research article ($n = 25$).

After abstract screening, 72 original research papers remained as being potentially relevant to this review. Of these, 3 papers were not obtainable, leaving 69 papers for full review. Data were extracted from each paper into an excel sheet, recording the main methods, results (up/down-regulation of miRs by outcome), and assessment of risk of bias, as described below.

2.2 Assessment of risk of bias for analysis and pooling of study results

Studies identified to be within the scope of this review where assessed for risk of bias by R.N., P.H., and D.G. A recent review of bias,[19] as well as the Cochrane Handbook for Systematic Reviews of Diagnostic Test Accuracy,[17,20,21] reports that there is consistent evidence of bias, with studies using a case-control design reporting higher accuracy of tests when compared with a cohort design. They also report a consistent association between the study outcome and the use of inappropriate reference standards or the biased recruitment of patients. The QUADAS-2 tool to assess quality of studies also highlights the potential bias inherent in the case-control design, patient recruitment, and use of reference standard.[16] For this review, the study design, patient selection, miR index test, and reference standard were the main aspects scrutinized for risk of bias for each outcome of interest. In addition, to study the potential risk of bias across studies, data were collected on how miRs were selected for each study and on all miRs reported to have been studied, to be able to compare and contrast negative and positive results across all studies, and to identify potential bias in the selection and selective reporting of miRs studied. Disagreements on risk of bias for individual studies were discussed and a final assessment made through consensus between these three authors. Pooling of results was planned for studies with the lowest potential for bias and for studies that were sufficiently homogeneous based on miR and disease being examined.

2.3 Mechanistic data of role of miRs in progression of cardiovascular diseases

A comprehensive search of the literature was undertaken for studies that presented data on the role of the miRs that were most studied and had the

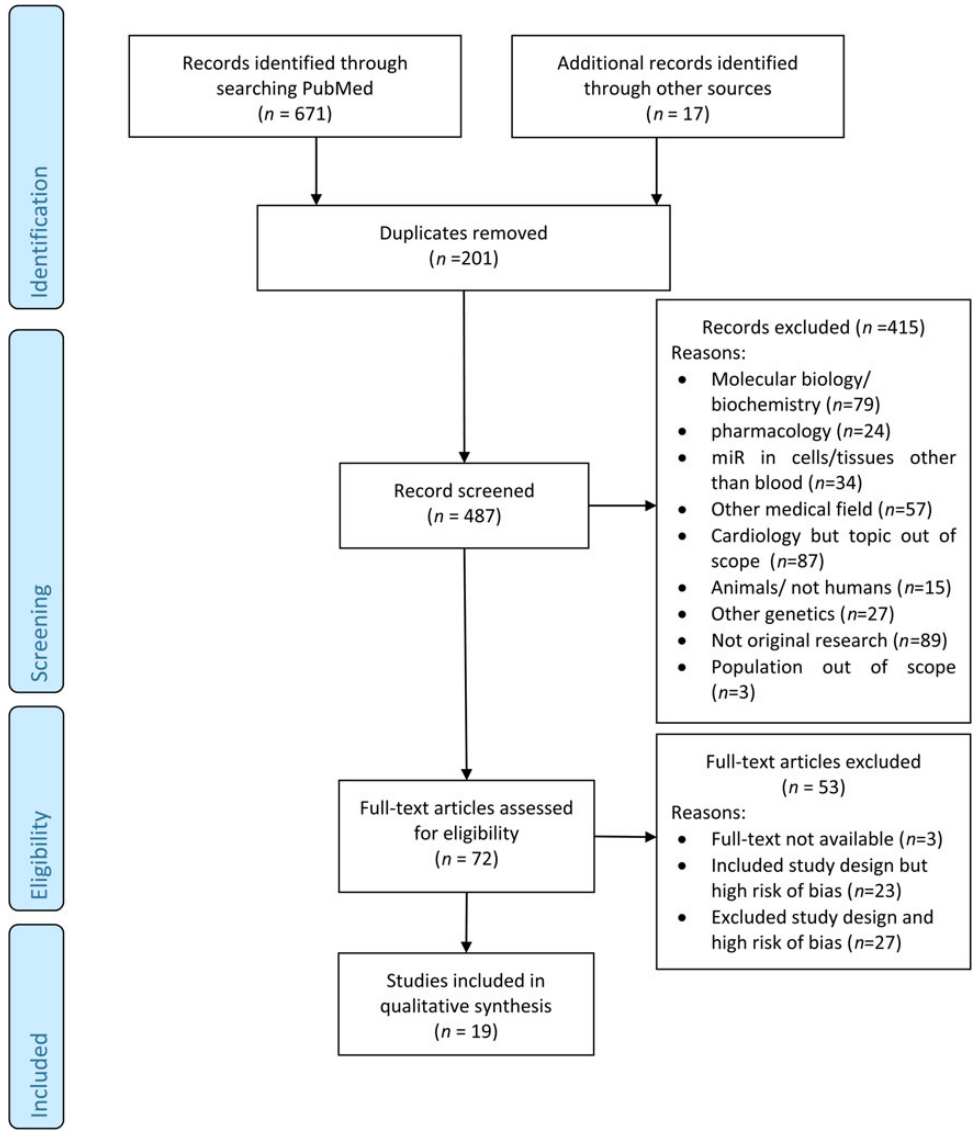

Figure 1 Flow diagram from study identification to inclusion.

most significant associations identified in this review. The last search was undertaken in PubMed on 12 February 2012 and included the following search terms:

(MicroRNAs[Mesh]) AND ('Coronary Artery Disease'[Mesh] OR 'Atherosclerosis'[Mesh] OR 'Myocardial Infarction'[Mesh] OR 'Acute Coronary Syndrome'[Mesh])
AND
((miR-1 OR miR-133 OR miR-133a OR miR-133b OR miR-145 OR miR-208 OR miR-208a OR miR-208b OR miR-499 OR miR-499a) NOT (mir[author]))

The mechanistic studies were reviewed and mechanisms related to cardiovascular disease progression described in the results section.

2.4 Target search

To better understand the relation between miRs and their function, we screened mirtarbase database for their RNA targets. We limited RNA species for *Homo sapiens* for which data were validated by reporter assay analysis, qPCR, and western blot analysis were available. We looked for shared targets to suggest common pathways.

3. Results

3.1 Part 1: clinical data on diagnostic potential

Overall, out of the 69 papers reviewed in full, 19 studies[22–40] were selected as having sufficient quality to assess the value of the miRs as potential biomarkers for cardiovascular disease. Data extracted from the included, missing,[41–43] and excluded studies, with an appropriate study design but insufficient quality[11,44–65] and with a less reliable study design and insufficient quality,[66–92] are available in Supplementary material online, *File 2*.

Due to the heterogeneity between included studies, in the study populations, settings, miRs and outcomes investigated, quantitative

Table 1 Overview of significant miRs identified in all included studies, organized by disease progression: non-CAD vs. CAD

Study	Number of patients	Tested miRs	Significant miRs	Statistical analysis
Gacoń *et al.*[24]	16 (−); 27 (+)[a]	miR-1, miR-16, miR-34a, miR-122, miR-124, miR-133a/b, miR-134, miR-208b, miR-375, miR-499	miR-34a, miR-124, miR-133a/b, miR-134 (+)	Unadjusted non-parametric Kolmogorov–Smirnov test[b,c]: miR-124 ($P < 0.025$); others ($P < 0.05$)
Gao *et al.*[25]	28 (−); 167 (+)	miR-145	miR-145 (−)	Multivariate linear regression[d]: $P < 0.0001$
Gao *et al.*[26]	100 (−); 155 (+)	miR-33a/b, miR-122, miR-370	miR-122, miR-370 (+)	Multivariate linear regression[d]: miR-122 ($P = 0.034$); miR-370 ($P = 0.022$)
Sun *et al.*[32]	36 (−); 31(+)	miR-126	–	Unadjusted Mann–Whitey *U* or Kruskal–Wallis test[b]: $P = 0.675$
Wang *et al.*[33]	92 (−); 154 (+)	miR-133a	miR-133a (+)	Multivariate linear regression[d]: $P < 0.001$
Zhu *et al.*[40]	54 (−); 56 (+)	miR-155	miR-155 (−)	Unadjusted Student's *t*-test[e]: $P < 0.001$

Patient numbers and the direction of regulation of significant miRs (in parentheses) are ordered from less to more severe disease.
Traditional risk factors: age, gender, body mass index, diabetes, hypertension, dyslipidemia, smoking; blood levels of glucose and/or lipids; other blood markers and medication as specified for each study separately.
[a]Patent IRA vs. occluded IRA.
[b]Patient characteristics: no significant differences for traditional risk factors and biomarkers.
[c]ROC AUC values are also available in the SF2 Study Quality table.
[d]Adjusted for traditional risk factors.
[e]No significant difference in traditional risk factors and medications taken, but significant difference in high-sensitivity C-reactive protein. However, miR-155 was not independent of age, hypertension, total C, HDL-C, LDL-C, high-sensitivity C-reactive protein, and smoking.

pooling of study results was deemed inappropriate, and therefore a qualitative summary by miRs and disease outcome/progression is presented below. Due to lack of clarity in the results of the groups compared and results presented in Li (2015),[29] this study was not included in the summaries below.

3.1.1 Non-CAD vs. CAD

Six original studies[24–26,32,33,40] (*Table 1*) investigated miRs and their value in distinguishing between CAD and non-CAD patients, questioning the need for recurrent diagnostic imaging.

miR-133a,[24,33] miR-134, miR-145,[25] miR-122, and miR-370[26] were associated with CAD presence, even after adjustment for other cardiovascular risk factors. Furthermore, their levels were positively correlated with the severity of CAD quantified by the Gensini score, in other words, may be used to predict both the presence and severity of coronary lesions in CAD patients. A single study reported miR-155[40] being associated inversely with complicated pro-atherogenic metabolic risk factors. In separate studies, miR-126,[32] miR-33a/b[26] as well as miR-1, miR-16, miR-122, miR-208b, miR-375, and miR-499[24] were not significantly down-regulated or up-regulated in CAD patients and so not suitable for discriminating CAD patients from patients without CAD.

3.1.2 Stable CAD vs. ACS

Three studies selected miRs to more accurately define stable CAD and ACS[25,31,37] (*Table 2*). miR-1, miR-21, and miR-499[31] proved to add to the diagnostic value of high-sensitive troponin (hs-Tn), a marker of cardiac injury, or in some cases be a better diagnostic biomarker than hs-Tn. miR-1, miR-499, and miR-21 significantly increased the diagnostic value in all suspected ACS patients, independent of clinical co-variants, including patient history and cardiovascular risk factors. All these miRs together with miR-145[25] were found to be associated with the severity of CAD. However, the biggest prognostic or diagnostic value was obtained with the combination of miR-132, miR-150, and miR-186,[37] even when compared with combined four classical biomarkers hsTnI, BNP, C-reactive protein (C-REACTIVE PROTEIN), and cystatin C.

3.1.3 Acute chest pain/UA vs. AMI

The major aim of five studies[22,34–36,38] (*Table 3*) was to identify superior diagnostic and prognostic markers for more accurate and timely diagnosis of UA and AMI. With the highest accuracy, miR-208a/b[22,34,35] stood out as the early detectable specific diagnostic marker of AMI when compared with cTnI or hs-cTnT. miR-133a,[34,35] miR-320a,[22] and miR-499[22,34,38] also proved to be sensitive and specific AMI diagnostic biomarkers. In contrast, two studies of miR-451[22,34] and single studies of miR-499,[35] miR-320,[36] and miR-16[34] showed no significant differences in the regulation of these miRs between comparison groups. A single population study investigated 18 miRs, with the results suggesting that only miR-126, miR-223, and miR-197[36] are associated with AMI disease risk. However, miR-126 and miR-197 were not tested in any of the other four studies and one other study questioning the finding of miR-223,[22] as they reported not having a significant difference in regulation between UA and AMI.

3.1.4 Non-STEMI vs. STEMI

Five of the studies also investigated the ability to detect differences in levels of miRs between patients with Non-STEMI vs. STEMI[22,24,25,35,39] (*Table 4*). Previously described as potential AMI diagnostic markers, miR-133a, miR-208b,[22,35] as well as miR-499 and miR-451,[22] and miR-134[24] levels were reported as higher in STEMI, when compared with non-STEMI. In contrast, two studies of miR-499[24,35] showed no significant difference in miR expression. A combination of miR-486 and miR-150[39] was reported to be sensitive and specific for the discrimination of non-STEMI cases from non-AMI controls, but not for discriminating non-STEMI from STEMI. It can be noted that STEMI

Table 2 Overview of significant miRs identified in all included studies, organized by disease progression: stable CAD vs. ACS

Study	Number of patients	Tested miRs	Significant miRs	Statistical analysis
Gao *et al.*[25]	26 (−); 141(+)[a]	miR-145	miR-145 (−)	Multivariate linear regression[b]: $P < 0.005$
Oerlemans *et al.*[31]	226 (−); 106 (+)[c]	miR-1, miR-21, miR-146a, miR-208a, miR-499	miR-1, miR-21, miR-146a, miR-208a, miR-499 (+)	Multivariate logistic regression (OR: 95% CI)[b,d,e]: miR-1 (1.30: 1.17–1.42); miR-21 (1.28: 1.18–1.39); miR-146a (1.14:1.08–1.21); miR-208a (1.16:1.03–1.30); miR-499 (1.28:1.18–1.40)
Zeller *et al.*[37]	48 (−); 49 (+)	miR-19a/b, miR-132, miR-140-3p, miR-142-5p, miR-150, miR-186, miR-210	miR-19a/b, miR-132, miR-140-3p, miR-142-5p, miR-150, miR-186, miR-210	Unadjusted Mann–Whitney test[f,e]: miR-19a/b ($P = 0.003$); miR-132 ($P = 0.005$); miR-140-3p ($P = 0.002$); miR-142-5p ($P = 0.001$); miR-150 ($P < 0.00001$); miR-186 ($P = 0.007$); miR-210 ($P < 0.0002$)

Patient numbers and the direction of regulation of significant miRs (in parentheses) are ordered from less to more severe disease.
Traditional risk factors: age, gender, body mass index, diabetes, hypertension, dyslipidemia, smoking; blood levels of glucose and/or lipids; other blood markers and medication as specified for each study separately.
[a]UA and non-STEMI combined.
[b]Adjusted for traditional risk factors.
[c]Twenty-four UA and 82 non-STEMI combined.
[d]Also adjusted for family and CVD history and cardiac hs-troponin T; overall, miR-1+ miR-499 + miR-21 showed the highest discriminatory power after adjustment (AUC = 0.94; 95% CI: 0.92–0.97).
[e]ROC AUC values are also available in the SF2 Study Quality table.
[f]Patients randomly selected from larger cohort studies and patient characteristics found comparable; miR-132, miR-150, and miR-186 showed the highest discriminatory power in validation (46 UA; 63 CAD patients) using logistic regression (AUC = 0.91; 95% CI: 0.84–0.98).

patients had significantly lower miR-145,[25] compared with the other groups indicating an altered expression of miR-145 in these patients. In contrast, miR-223 and miR-320a,[22] as well as miR-1,[24,35] and 7 additional miRs[24] provided no added diagnostic value for discriminating between non-STEMI and STEMI patients when combined with cTnT or hs-cTnT.

3.1.5 Mortality

The seven selected studies[22,23,27,28,30,35,36] (*Table 5*) did not identify miRs which significantly predicted long-term mortality; however, mid-term and short-term mortality could be predicted to a certain extent.

miR-208b[22,35] is one of few significant mortality predicting biomarkers, which remained significant after adjustment for age and gender.

miR-133a[35] levels were significantly related to all-cause mortality, with a single study reporting a 2.5-fold higher risk of death in the fourth quartile compared with patients in the first three quartiles ($P = 0.011$). It remained significant after adjustment for age and gender, but not after further adjustment for hs-TnT levels on admission. However, in contrast, another study found no difference in expression for miR-208b[27] or miR-133a.[22]

miR-328[28] was found to increase in patients who experienced heart failure or death within 6 months in the only study testing this miR. In contrast, miR-134[28,30] and miR-223[22,36] were investigated in two different studies each with opposing results in terms of miR expression.

All three studies testing miR-499[22,27,35] and two studies testing miR-93,[30,36] miR-320,[22,36] or miR-451[22,35] found no significant difference in expression in those that survived vs. those that died. In addition, 22 other miRs were tested once and did not show any difference in level of their expression between comparison groups.

3.2 Cardiovascular disease progression

Some miRs, including miR-133a/b (5 studies[22,24,33–35]), miR-208a/b (6 studies[22,24,27,31,34,35]), and miR-499 (7 studies[22,24,27,31,34,35,38]) have been well studied and found to be significant diagnostic and/or prognostic markers across different cardiovascular disease progression stages (*Table 6*), in most studies outperforming cTnT or hs-cTnT. miR-1 is a potential biomarker of ACS, with higher sensitivity for AMI.[31,34,35] Another potential biomarker of ACS is miR-145,[23,26] with higher sensitivity for STEMI and worse outcome of AMI.

Mid- and short-term mortality prediction was reported, with contrasting results for miR-133a[22,35] and miR-208b.[22,27,35]

No firm conclusions about the potential diagnostic value of miR-21,[31,36] miR-126,[32,36] miR-134,[24,28] miR-146a/b,[31,36] miR-150,[36,37,39] and miR-486[39] can be drawn because of lack of studies across different cardiovascular disease progression stages and contrasting results between studies. In contrast to miRs which were found to be associated with one or more stages in cardiovascular disease progression, miR-122 was found to be non-specific in all studies,[24,26,36] as were miR-16,[24,34] miR-223,[22,36] and miR-320(a).[22,36]

3.3 Part 2: mechanistic data of role of miRs in progression of cardiovascular diseases

After review for relevance by two study authors (P.H. and R.N.), the studies presenting mechanistic data are described below. We searched mechanistic data for miRs with significant associations across different cardiovascular disease progression states and therefore have the highest potential as biomarkers. Several other recent reviews have discussed roles of miRs in atherosclerosis and cardiovascular diseases.[14,93–99] Functions of miRs are summarized in *Figure 2*.

Table 3 Overview of significant miRs identified in all included studies, organized by disease progression: acute chest pain/UA vs. AMI

Study	Number of patients	Tested miRs	Significant miRs	Statistical analysis
Devaux *et al.*[22]	931 (−); 224 (+)	miR-133a, miR-208b, miR-223, miR-320a, miR-451, miR-499	miR-208b, miR-320a, miR-499 (+)	Unadjusted Mann–Whitey *U* or Kruskal–Wallis test[a,b]: miR-208b ($P < 0.001$); miR-320a ($P = 0.031$); miR-499 ($P < 0.001$)
Wang *et al.*[34]	33 (−); 33 (+)	miR-1, miR-16, miR-133a, miR-208a, miR-451, miR-499	miR-1, miR-133a, miR-208a, miR-499 (+)	Unadjusted ROC (AUC: 95% CI)[c]: miR-1 (0.85: 0.75–0.94); miR-133a (0.87: 0.77–0.96); miR-208a (0.97: 0.92–1.00); miR-499 (0.82: 0.72–0.93)
Widera *et al.*[35]	117 (−)[d]; 327 (+)	miR-1, miR-133a/b, miR-208a/b, miR-499	miR-1, miR-133a, miR-208b (+)[e]	Unadjusted Mann–Whitey *U* test[f,d]: miR-1 ($P < 0.001$); miR-133a ($P < 0.001$); miR-208b ($P = 0.044$)
Zampetaki *et al.*[36]	773 (−)[e]; 47 (+)	miR-7b/e, miR-21, miR-24, miR-25, miR-28-3p, miR-93, miR-122, miR-126, miR-140, miR-146b, miR-150, miR-191, miR-197, miR-223, miR-320, miR-342-3p, miR-454, miR-486	miR-126 (+); miR-197, miR-223 (−)	Multivariate Cox regression (HR: 95% CI)[g,e]: miR-126 (2.69: 1.45–5.01, $P = 0.002$); miR-197 (0.56: 0.32–0.96, $P = 0.036$); miR-223 (0.47: 0.29–0.75, $P = 0.002$)
Zhang L *et al.*[38]	85 (−); 142 (+)	miR-499	miR-499 (+)	Unadjusted ROC (AUC: 95% CI)[f]: 0.86: 0.81–0.91

Patient numbers and the direction of regulation of significant miRs (in parentheses) are ordered from less to more severe disease.
Traditional risk factors: age, gender, body mass index, diabetes, hypertension, dyslipidemia, smoking; blood levels of glucose and/or lipids; other blood markers and medication as specified for each study separately.
[a]Several significant differences in traditional risk factors; also miRs no longer significant predictors when multivariate regression included troponins.
[b]ROC AUC values are also available in the SF2 Study Quality table.
[c]Patient traditional risk factors comparable, but significant difference in HDL and WBC.
[d]Only UA patients, but large clinical overlap between UA and AMI.
[e]Population-based study: all adults ≥40 years; and adjusted for CVD history, other miRs, waist-to-hip ratio, C-reactive protein, and fibrinogen.
[f]Patient characteristics: significant differences between traditional risk factors not presented/reported for these patient groups.
[g]Adjusted for traditional risk factors and biomarkers.

miR-1 prevented endothelial permeability in apoE KO mice[100] and induced angiogenesis by repressing mRNA encoding seryl-tRNA synthetase.[101] Its effect on angiogenesis was opposite to that of miR-206.[101,102] In addition, miR-1-transfected embryonic stem cell enhanced cardiac myocyte differentiation and inhibited apoptosis by modulating the PTEN/Akt pathway in the infarcted heart.[103] miR-1 expression depended on SERCA2. Indeed, SERCA2a gene therapy of failing hearts restored miR-1 expression by an Akt/FoxO3A-dependent pathway, which is associated with normalized expression of sodium–calcium exchanger 1 (NCX) resulting in improved cardiac function.[104]

miR-133 suppressed angiogenesis properties of endothelial cells such as proliferation rate, cell viability, and migration activity by targeting VEGFR2 and FGFR1.[105] miR-133 decreased when vascular smooth muscle cells (VSMCs) were primed to proliferate *in vitro* and following vascular injury *in vivo*, whereas it increased when VSMCs were coaxed back to quiescence *in vitro* and *in vivo*. miR-133 loss- and gain-of-function experiments showed that miR-133 plays a mechanistic role in VSMC growth. Accordingly, adeno-miR-133 reduced but anti-miR-133 exacerbated VSMC proliferation and migration *in vitro* and *in vivo*, by suppressing the transcription factor Sp-1 expression.[106] miR-133 and miR-1, which belong to the same transcriptional unit, were decreased in mouse and human models of cardiac hypertrophy. *In vitro* overexpression of miR-133 or miR-1 inhibited cardiac hypertrophy. In contrast, suppression of miR-133 by 'decoy' sequences induced hypertrophy. *In vivo* inhibition of miR-133 by a single infusion of an antagomir caused marked and sustained cardiac hypertrophy. RhoA, a GDP–GTP exchange protein regulating cardiac hypertrophy; Cdc42, a signal transduction kinase implicated in hypertrophy; and Nelf-A/WHSC2, a nuclear factor involved in cardiogenesis were identified as specific targets of miR-133.[107]

miR-145 and miR-143 were found to be co-transcribed in multipotent mouse cardiac progenitors before becoming localized to smooth muscle cells; including neural crest stem cell-derived VSMCs. miR-145 and miR-143 were down-regulated in injured or atherosclerotic vessels containing proliferating, less differentiated smooth muscle cells. Restoration of miR-145 in balloon-injured arteries via adenovirus miR-145 inhibited neointimal growth by inducing VSMC differentiation marker genes such as SM alpha-actin and calponin by up-regulating Kruppel-like factor (KLF)-5 and its downstream signalling molecule, myocardin. Transient decrease in miR-145 expression 3 days post-myocardial infarction (MI) was associated with an increase in KLF-5 and a decrease in myocardin. *In vivo* delivery of a miR-145 antagomir 1 day prior to and 2 and 6 days after MI, suppressed expression of KLF5 protein, decreased myofibroblast formation, and increased scar size.[108] miR-145 was necessary for myocardin-induced reprogramming of adult fibroblasts into smooth muscle cells and sufficient to induce differentiation

Table 4 Overview of significant miRs identified in all included studies, organized by disease progression: non-STEMI vs. STEMI

Study	Number of patients	Tested miRs	Significant miRs	Statistical analysis
Devaux *et al.*[22]	179(−); 45(+)	miR-133a, miR-208b, miR-223, miR-320a, miR-451, miR-499	miR-133a, miR-208b, miR-451, miR-499 (+)	Unadjusted Mann–Whitey *U* or Kruskal–Wallis test[a]: miR-133a ($P = 0.004$); miR-208b ($P = 0.001$); miR-499 ($P = 0.001$); miR-451 ($P = 0.44$)
Gacon *et al.*[24]	27 (−); 16 (+)	miR-1, miR-16, miR-34a, miR-122, miR-124, miR-133a/b, miR-134, miR-208b, miR-375, miR-499	miR-134 (+)[b]	Unadjusted non-parametric Kolmogorov–Smirnov test[b,c]: $P < 0.025$
Gao *et al.*[25]	106 (−)[d]; 35 (+)	miR-145 (−)	miR-145 (−)	Multivariate linear regression[e]: $P < 0.008$
Widera *et al.*[35]	131 (−); 196 (+)	miR-1, miR-133a/b, miR-208a/b, miR-499	miR-133a (−); miR-208a (+)	Unadjusted Mann–Whitey *U* test[a]: miR-133a ($P < 0.001$); miR-208a ($P < 0.001$)
Zhang R *et al.*[39]	45 (−); 65 (+)	miR-150, miR-486	miR-150, miR-486 (+)	Unadjusted independent sample *t*-tests or Mann–Whitney *U* test[a]: miR-150 ($P = 0.016$); miR-486 ($P = 0.015$)

Patient numbers and the direction of regulation of significant miRs (in parentheses) are ordered from less to more severe disease.
Traditional risk factors: age, gender, body mass index, diabetes, hypertension, dyslipidemia, smoking; blood levels of glucose and/or lipids; other blood markers and medication as specified for each study separately.
[a]Patient characteristics: significant differences between these groups not presented/reported.
[b]Potentially confounded by extent of coronary stenosis; significant differences between patient groups only for pain onset and hs-TnTmax, not for other characteristics listed in *Table 1*.
[c]ROC AUC values are also available in the SF2 Study Quality table.
[d]UA and non-STEMI combined.
[e]Adjusted for traditional risk factors.

of multipotent neural crest stem cells into VSMCs. Furthermore, miR-145 and miR-143 cooperatively targeted a network of transcription factors, including KLF-4, myocardin, and ELK1, a member of ETS oncogene family, to promote differentiation and repress proliferation of smooth muscle cells. These findings demonstrated that miR-145 can direct the smooth muscle fate and that miR-145 and miR-143 function to regulate the quiescent vs. proliferative phenotype of smooth muscle cells.[109] In addition, miR-145 and miR-143 act as communication molecules between smooth muscle and endothelial cells to enhance the angiogenic and vessel stabilization properties of endothelial cells.[110] Angiotensin II[111,112] down-regulated miR-145 and reduced KLF4 and myocardin expression in human coronary arterial smooth muscle cells. Overexpression of miR-145 and treatment with valsartan reversed KLF4 and myocardin protein expression and improved vascular injury induced by balloon injury. Mechanical stretch suppressed miR-145 expression by activating extracellular signal-regulated kinase 1/2 and up-regulating angiotensin-converting enzyme to alter VSMC phenotype, with reduced expression of contractile markers. Moreover, transcoronary gradients of miR-145-5p, and of miR-126-3p, correlated with the extent of thin-cap fibroatheromas suggesting that instable plaques may affect the local uptake or degradation of these miRs.[113]

Data discussed above suggested a protective effect of miR-145, but other studies suggested an opposite effect. Indeed, miR-143/145 deficiency *per se* resulted in increased hepatic and vascular ABCA1 expression and significant reduction of atherosclerosis.[114] Moreover, down-regulation of miR-145 protected against the development of pulmonary arterial hypertension (PAH). In patient samples of heritable PAH and idiopathic PAH, miR-145 is expressed in remodelled vessels and mutations in BMPR2 lead to up-regulation of miR-145 in mice and PAH patients.[115]

A miR-208–Mef2 axis was found to drive the decompensation of right ventricular function in pulmonary hypertension. miR-208 inhibition, as well as tumour necrosis factor-α, activated the complex mediator of transcription 13/nuclear receptor co-repressor 1 axis, which in turn promoted Mef2 inhibition, closing a self-limiting feedback loop, driving the transition from compensating phase of right ventricle hypertrophy towards de-compensation phase.[116] Analysis of mice lacking miR-208a indicated that miR-208a was required for proper cardiac conduction and expression of the cardiac transcription factors homeodomain-only protein and GATA4 and the gap junction protein connexin 40.[117]

Heart function was restored in rodents by reprogramming non-myocytes into cardiomyocytes, by expressing transcription factors [GATA4, HAND2, myocyte-specific enhancer factor 2C (MEF2C), and T-box 5 (TBX5)] and miRs (miR-1, miR-133, miR-208, and miR-499) that control cardiomyocyte identity. Stimulating cardiomyocyte dedifferentiation and proliferation by activating mitotic signalling pathways involved in embryonic heart growth represents a complementary approach for heart regeneration and repair.[118] miR-499 played an inhibiting role in the mitochondrial apoptosis pathway and had protective effects against H_2O_2-induced injury in cardiomyocytes.[119]

3.4 Part 3: RNA targets of miRs in progression of cardiovascular diseases

In addition, we searched targets for these miRs which could give us further information about mechanistic action and potential additive effects of some miRs by sharing target RNA species. Supplementary material

Table 5 Overview of significant miRs identified in all included studies, organized by disease progression: survival vs. death

Study	Number of patients	Tested miRs	Significant miRs	Statistical analysis
Devaux *et al.*[22]	1053 (−); 102(+)[a]	miR-133a, miR-208b, miR-223, miR-320a, miR-451, miR-499	miR-208b (+)	Unadjusted ROC (AUC: 95% CI)[a]: 0.67: 0.52–0.81
Dong *et al.*[23]	224(−); 22(+)[b]	miR-145	miR-145 (+)	Multivariate Cox regression (HR: 95% CI)[b,c]: 5.63: 1.99–15.91, $P = 0.0012$
Goretti *et al.*[27]	446(−); 64(+)[d]	miR-208b, miR-499	–	Multivariate linear regression[d]: ORs and 95% CI include 1
He *et al.*[28]	276 (−); 83 (+)[e]	miR-134, miR-328	miR-134, miR-328 (+)	Multivariate logistic regression (OR: 95% CI)[e]: miR-134 (2.28: 1.03–11.32, $P = 0.013$); miR-328 (7.35: 1.07–17.83, $P = 0.004$)
Matsumoto *et al.*[30]	21(−); 19(+)[f]	miR-18a, miR-93, miR-125a-5p, miR-134, miR-155, miR-190b, miR-192, miR-212, miR-223, miR-331-3p, miR-380	miR-155 (+), miR-380	Mann–Whitney *U* test[f]; $P < 0.05$
Widera *et al.*[35]	410 (−); 34 (+)[g]	miR-1, miR-133a/b, miR-208a/b, miR-451, miR-499	miR-133a, miR-208b (+)	Multivariate Cox regression (HR: 95% CI)[g,c]: miR-133a (3.5: 1.8–7.0, $P = 0.001$); miR-208b (2.7: 1.4–5.3, $P = 0.006$)
Zampetaki *et al.*[36]	21 (−); 26 (+)[h]	miR-7b/e, miR-21, miR-24, miR-25, miR-28-3p, miR-93, miR-122, miR-126, miR-140, miR-146b, miR-150, miR-191, miR-197, miR-223, miR-320, miR-342-3p, miR-454, miR-486	miR-126 (+); miR-223 (−)	Multivariate Cox regression (HR: 95% CI)[h]: miR-126 (2.70: 1.21–6.05, $P = 0.016$); miR-223 (0.37: 0.2–0.7, $P = 0.002$)

Patient numbers and the direction of regulation of significant miRs (in parentheses) are ordered from less to more severe disease.
Traditional risk factors: age, gender, body mass index, diabetes, hypertension, dyslipidemia, smoking; blood levels of glucose and/or lipids; other blood markers and medication as specified for each study separately.
[a]All-cause 30-day mortality; patient characteristics: significant differences between these groups not presented/reported; no miRs significantly predicted mortality when univariate Cox proportional hazard analysis undertaken
[b]Cardiac death at 1 year; hazard regression analysis adjusted for age, circulating miR-145, cTnI, CK-MB, LVEF, eGFR, and NT-proBNP.
[c]ROC AUC values are also available in the SF2 Study Quality table.
[d]AMI only 6-year mortality, adjusted for traditional risk factors.
[e]Heart failure or cardiogenic death within 6 months combined, adjusted for age, gender, current smoking, hs-cTnT, NTproBNP, and time from AMI onset to sampling.
[f]Cardiac death at 1-year post-hospital discharge following MI; to adjust for confounding patients were selected from larger cohort using propensity score by logistic regression of traditional risk factors and previous MI, Killip class at admission, infarct size, reperfusion therapy, and medication at discharge.
[g]All-cause 6-month mortality; significant difference in survival adjusted for age and gender. However, no significant miRs remained when Cox regression analysis included hsTnT.
[h]Non-fatal vs. fatal MI within 10 years of study; adjusted for traditional risk factors and CVD history, other miRs; potentially confounded by death occurring in first vs. last 5 years for miR-126.

online, *Table S3* gives an overview of all significant RNA targets for which data validated by reporter assay analysis, qPCR, and western blot analysis were available. *Table 7* summarizes RNA targets which are shared between at least two miR families.

We searched for common targets between these miRs. ETS proto-oncogene 1, transcription factor (ETS1), involved in stem cell development, cell senescence, and death, and tumour development was shared between miR-1, miR-145, miR-208, and miR-499. It is implicated regulation of platelet-derived growth factor receptor-alpha gene transcription in VSMCs.[120]

miR-1 and miR-133 shared 4 RNA targets: hyperpolarization-activated cyclic nucleotide-gated potassium channel 4 (HCN4), LIM and SH3 protein 1 (LASP1), purine nucleoside phosphorylase (PNP), and transgelin 2 (TAGLN2). HCN4 channels regulate pacemaker activity.[121,122] An increase in HCN (HCN2 and HCN4) channel activity in hypertrophied myocytes prolonged the re-polarization of the ventricular action potential, possibly linking an up-regulation of ventricular depolarizing current $I(f)$ and a diminished re-polarization reserve in cardiac hypertrophy.[123] The association between the chemokine receptors and LASP-1 is crucial for regulation of migration of inflammation cells.[124] PNP is crucial under energy-deprived conditions for the cell to metabolize adenosine during ATP degradation and as such for regulation growth of activated T cells.[125] miR-133a was found to directly bind to the 3′ UTR of TAGLN2 mRNA and thereby suppressed expression at both transcriptional and translational levels, affecting differentiation of smooth muscle cells. Next, TAGLN2 knockout was used to reveal that TAGLN2 modulated hypoxia-induced apoptosis via caspase-8 apoptotic pathway.[126]

miR-133 shares two other RNA targets with miR-145: fascin actin-bundling protein 1 (FSCN1) and insulin-like growth factor 1 receptor (IGF1R). FSCN1 plays a critical role in cell migration, motility, adhesion, and cellular interactions. Several studies have shown that the interaction of miR-145 and miR-133 with FSCN1 is crucial to inhibit migration and invasion of cancer cells.[127–129] To our knowledge, it has not yet been associated with atherosclerosis and cardiovascular diseases. Reduction of miR-133a expression was associated with lower IGF1R levels and suppression of VSMC growth through modulation of the Akt/FOXO3a pathway.[130,131] The antidiabetic hormone glucagon-like peptide-1 induced formation of new elastic fibres in human cardiac fibroblasts after cross-activation of IGF1R, possibly contributing to beneficial remodelling of the human heart after MI.[132]

Finally, miR-145 and miR-208 target cyclin-dependent kinase inhibitor 1A (CDKN1A; or p21 or p21Cp1), a regulator of cell cycle

Table 6 Overview of significant miRs identified in all included studies, organized by disease progression: cardiovascular disease progression

miR[a]	CAD	ACS	AMI	STEMI	Mortality	Study reference
miR-1	No[24]	+[31]	+[34,35]	No[24]	No[35]	24,31,34,35
miR-21	–	+[31]	No[36]	–	No[36]	31,36
miR-122	No[24]; +[26]	–	No[36]	No[24]	No[36]	24,26,36
miR-126	No[32]	–	+[36]	–	+[36]	32,36
miR-133a/b	+[24,33]	–	No[22]; +[34,35]	No[24]; +[22,35]	No[22]; +[35]	22,24,33–35
miR-134	+[24]	–	–	+[24]	No[30]; +[28]	24,28,30
miR-145	+[25]	+[25]	–	+[25]	+[23]	23,25
miR-146a/b	–	+[31]	No[36]	–	No[36]	31,36
miR-150	–	+[37]	No[36]	+[39]	No[36]	36,37,39
miR-208a/b	No[24]	+[31]	+[22,34,35]	No[24]; +[22,35]	No[27]; +[22,35]	22,24,27,31,34,35
miR-320(a)	–	–	No[36]; +[22]	No[22]	No[22,36]	22,36
miR-486	–	–	No[36]	+[39]	No[36]	36,39
miR-499(a)	No[24]	+[31]	No[35]; +[22,34,38]	No[24,35]; +[22]	No[22,27,35]	22,24,27,31,34,35,38

Patient numbers and the direction of regulation of significant miRs (in parentheses) are ordered from less to more severe disease.
[a]miRs were only included in this table if they were studied in at least two different studies and across at least two areas of disease progression. miRs presented have been reported as having a significant difference in miR expression (+) or no difference in miR expression (No) between patients compared in the studies, with each study reference number included as a subscript. However, miR-16 and miR-223 were not added since no studies reported a significant difference of miR expression between patient groups.

progression at G1. Gene silencing studies demonstrated that disturbed flow induced endothelial cell senescence via a p53–p21 signalling pathway, resulting in reduced migration, and thus may be defective for arterial repair.[133] The microRNA-155/FOXO3a signalling pathway and induction of induction of FOXO3a targets, p21 and p27(kip1), have been implied in global remodelling of the vascular stem cell niche in bone marrow of diabetic patients.[134]

4. Discussion

Our review identified miR-1, miR-133a/b, miR-145, miR-208a/b, and miR-499(a) as the most promising miR biomarkers and/or prognostic markers for progressing stages of cardiovascular disease. Interestingly 4 of those miRs (miR-1, miR-133, miR-208, and miR-499) were found to control cardiomyocyte identity.[118] In addition, they share several other functions such as regulation of endothelial function and angiogenesis (miR-1, miR-133), VSMC differentiation (miR-133, miR-145), communication between vascular smooth muscle and endothelial cell to stabilize plaques (miR-145), and apoptosis (miR-1, miR-133, miR-499), which are crucial processes in the pathogenesis of cardiovascular disease. Finally, we identified shared RNA targets linking them to common functions. These functions further support their clinical relevance as diagnostic markers.

Although 19 studies were included in the review, no meta-analysis was undertaken due to the heterogeneity of the studies, including the differences across miRs and diseases studied, and also because of important differences and limitations in study design including: clinical settings, population characteristics, sample collection and handling, and qPCR normalization methods. These limitations led the research team to provide an overview of the limitations and highlight examples of good practice identified in this review, to inform future research on the methods to consider when undertaking and reporting properly designed miR biomarker studies.

4.1 Participant characteristics and comparison groups

Proper comparison groups are needed to be able to make a differential diagnosis. Comparing patients with disease to healthy controls has been shown to overestimate the accuracy of the diagnostic test,[19] and in practice, clinicians are assessing and comparing patients with chest pain/disease and so need a diagnostic tool that will more accurately distinguish between diseased patients. Most studies that were excluded from the analysis compared patients with healthy controls and did not adjust for differences in metabolic profiles of patients and healthy controls, increasing the likelihood of obtaining more significant differences in miR expression between patients and controls but without adding clinical value in discriminating between patients who present with similar symptoms and metabolic profiles. Since diagnostic studies are not often able to be designed as randomized clinical trials, it is important for these studies to provide clear phenotyping of patients with known potential confounders and risk factors for disease.

Too often, adjustment for comorbidities and medication is lacking.[135] However, information on medication is crucial in view of a recent discovery that miRs determine the efficacy of drugs, which has given rise to the field of 'miRNA pharmacogenomics' through 'Pharmaco-miRs'. Indeed, miRs play a significant role in pharmacogenomics by down-regulating genes that are important for drug function.[136] In addition, medication affects miR expression. Statins were

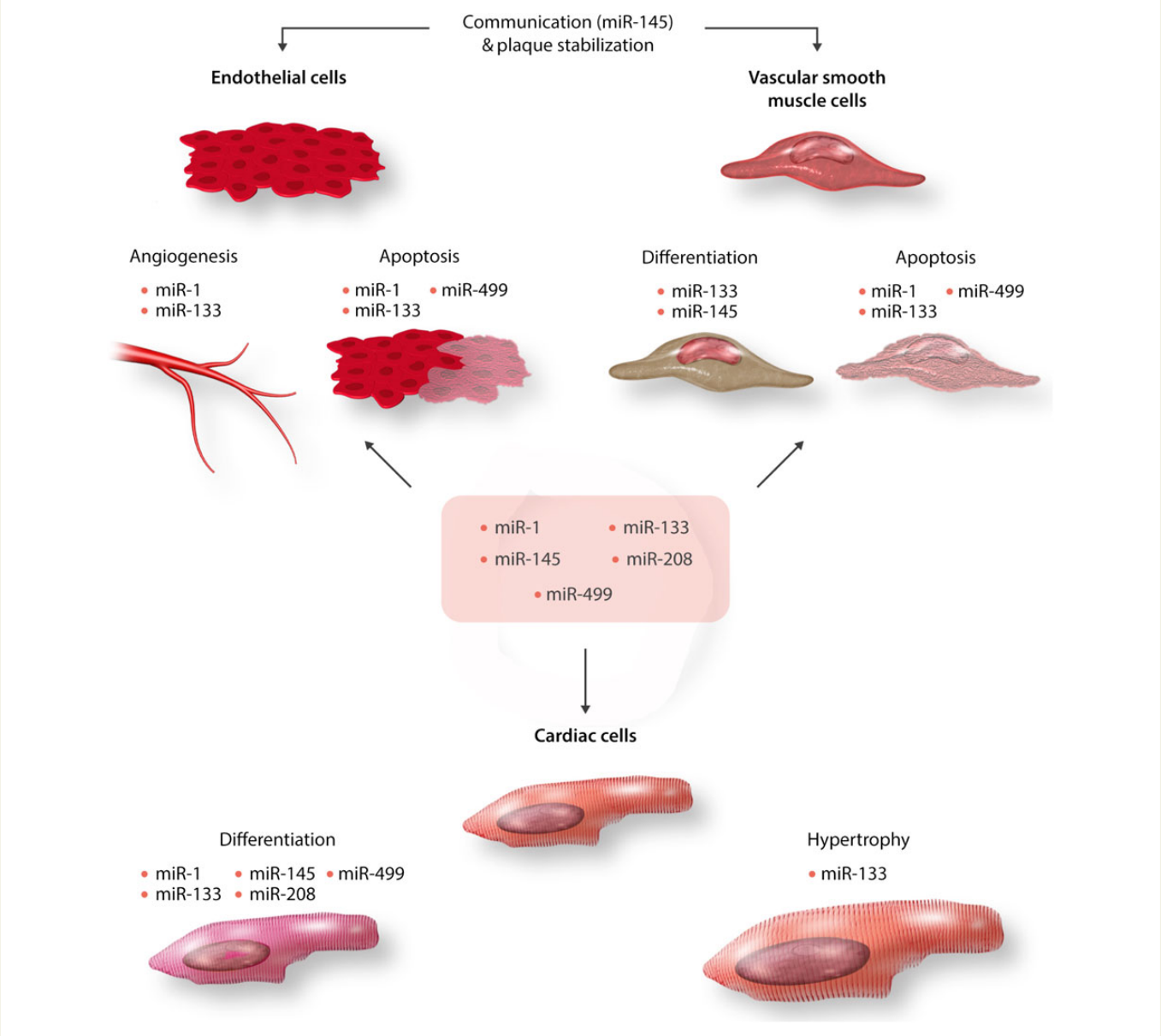

Figure 2 Function of miRs. We identified miR-1, miR-133, miR-145, miR-208, and miR-499 as significant diagnostic and/or prognostic markers across different cardiovascular disease progression stages. They regulate endothelial function and angiogenesis (miR-1, miR-133), VSMC differentiation (miR-133, miR-145), communication between vascular smooth muscle and endothelial cell to stabilize plaques (miR-145), and regulate apoptosis in endothelial and VSMCs (miR-1, miR-133, miR-499), cardiac myocyte differentiation (miR-1, miR-133, miR-145, miR-208, miR-499), and repress cardiac hypertrophy (miR-133).

Table 7 Overview of RNA targets shared by at least two miR families

	CDKN1A	ETS1	FSCN1	HCN4	IGF1R	LASP1	PNP	TAGLN2
miR-1-3p		Y		Y		Y	Y	Y
miR-133a-3p			Y			Y	Y	Y
miR-133b			Y	Y				Y
miR-145-5p	Y	Y	Y		Y			
miR-208a-3p	Y	Y						
miR-208b-3p	Y							
miR-499a-5p		Y						

found to change the expressions of 22 whole blood miRs and 19 plasma miRs.[137] Willeit and Zampetaki *et al.*[138] found that plasma levels of platelet miRs, such as miR-223 and miR-191, and others, such as miR-126 and miR-150, decreased on platelet inhibition. It has also been shown that heparin can alter miRs in plasma, and therefore blood samples should be collected prior to heparin administration.[139]

As the topic of miR in cardiology is still new and the technology is expensive, several selected studies enrolled small numbers of subjects, also limiting the generalizability of the findings.

4.2 Sample collection and handling

Divergence between studies may also be due to differences in sample collection and handling. However, sufficient information to reproduce and ensure high-quality sample collection and handling is often scarce in publications. In all steps when collecting whole blood, measures should be taken to prevent contamination with mi(RNA) from intact cells and to prevent haemolysis. Hence, we recommend the National Cancer Institute's Early Detection Research Network standard operating procedures for the collection of serum and plasma.[140] The whole blood should be processed immediately. In the preparation of plasma, anticoagulants such as EDTA and citrate should be used. Heparin should be avoided because it is known to inhibit cDNA synthesis and PCR.[141] Haemolysis should be screened by measuring oxyhaemoglobin absorbance at 414 nm.[142] Once prepared and before freezing, plasma or serum should be centrifuged for 5 min at 3000 *g* to remove cells.

4.3 qPCR normalization methods

Divergence between studies may also be due to differences in qPCR normalization methods. These methods rely on two standard assumptions: (i) the majority of features do not vary between samples, and (ii) the proportions of up- and down-regulated expressions are approximately equal. To compensate for the lack of robustness in the existing methods, the least-variant set (LVS) normalization for mRNA arrays was developed based on data-driven housekeeping genes.[143] For normalization of miR data, one can (i) identify and use several stably expressed reference miRs, or even better (ii) use the global mean expression value of all commonly expressed miRs in a given sample as normalization factor.[144–147] However, only one study included in this review presented the reference miR expression levels—visually confirming that the level of the reference miR did not change between comparison groups.[33] One should avoid using larger small RNA species such as U6 because they have a different biogenesis pathway and may not be secreted or protected in biofluids in the same way as miRs are.[148,149] Furthermore, the use of exogenous spike-in controls to obtain standardized normalization has been considered, with seven of the included studies in this review also using an exogenous spiked in miR reference (see the SF2 Study Quality table). Recently, Roberts *et al.*[150] compared the use of different normalization strategies for the quantification of extracellular miRs in mouse serum. The levels of the putative endogenous miRs were positively correlated with the external spike-in control, indicating that variations in the latter reflect changes in endogenous miR abundance. Furthermore, the external spike-in control was the least variable miR across all samples in the RT-qPCR array study, and between experimental groups in the time course study thus validating this normalization approach. Additionally, more miRs were statistically significant, and greater fold changes observed, when the data were normalized to the spike-in control as opposed to the average Cq. The ratio of up- and down-regulated miRs was more balanced when the data were normalized to the average Cq. But, the observation of a global shift in miR levels suggests that this 'balance' may be artificial.[150] Several companies (e.g. Exiqon) have developed a set of synthetic spike-in RNAs which can be used to monitor the efficiency of RNA isolation, cDNA synthesis, and PCR amplification and to reveal potential presence of nucleases.

4.4 Selection of miRs

For this systematic review, we were only able to access data which are available in the public domain[151]; it is possible that other miRs were measured in the studies and were not reported, which is supported by the reporting of nine included studies that only present data on one or two miRs. It is also possible that other miRs studies with negative results were not published at all, adding to the potential bias in identifying miRs with the best potential to support diagnosis or prognosis of cardiovascular disease. There is also inherent bias in the selection of the miRs studied, as the field is still developing, only few of the known miRs have been studied. In all but four cases in the included studies, the decision was made on insights from the previous literature, so few studies have used array technology to screen vast numbers of miRs and usually have done so for a very small number of participants. Because there are so many potential miRs to study, only a few studies have replicated similar combinations of miRs in comparable cohorts. In this review, miR-1, miR-133a/b, miR-208a/b, and miR-499(a) were studied in four or more separate studies but across different disease comparison groups and with contrasting results. Although we excluded case-control studies for reasons explained above, we want to acknowledge the importance of the pioneer studies because they have been driving miR selection. In particular, miR-1,[67,68,77,82] eventually in combination with miR-133 and miR-499,[70] and miR-133, miR-208, and miR-499 have been studied extensively.[66,69,72,88,91]

Several studies[24,31,37] also suggested that the diagnostic values of clusters of miRs may be higher than that of any single miR. However, cluster analysis does not provide information as to how a certain miR cluster would translate into pathogenesis or altered cellular functions in the investigated pathology.

Our approach of collating several studies allowed us to provide a list of potentially useful miRs for diagnosis of cardiovascular disease; however, a self-selection bias that arises from scientific trends to investigate similar or same hot-spot miRs by several groups cannot be excluded. Nevertheless, we conclude that such assumptions are inherently present in any secondary literature study and regard it as a benefit of our review process to make these assumptions explicit and traceable.

To prevent selective publication and selective reporting of miR research outcomes, data-sharing plans are needed, as recently proposed by the International Committee of Medical Journal Editors.[152] Sharing data will increase confidence and trust in the conclusions drawn from clinical studies. It will enable the independent confirmation of results, an essential tenet of the scientific process. It may also make progress more efficient by avoiding unwanted repetition in case of reporting high-quality negative results.

4.5 Mirs in cells or tissues

In our review, we focused on miRs in plasma and serum and miRs examined in cells or tissues other than blood were excluded. Indeed, several miR-based studies have utilized tissues/cells for identifying miRs. But tissues other than blood (and eventually urine) are much less accessible and therefore less useful for biomarker analysis. In addition, several of our identified miRs are claimed to have tissue specificity. miR-1 is the most abundant miR specific for cardiac and skeletal muscle

and functions as a regulator of differentiation and proliferation during cardiogenesis[153] as well as a regulator of cardiomyocyte growth in the adult heart. In human hearts of patients who had died of MI, miR-1 was up-regulated in remote myocardium when compared with infarcted tissue or healthy adult heart.[154] Adachi *et al.* performed a miR array analysis in various human tissues to identify heart-specific miRs and found miR-499 almost specifically expressed in the heart, concluding that miR-499 might serve as an additional promising biomarker of MI.[58,66] miR-133, which is transcribed from the same chromosomal loci as miR-1, enhances myoblast proliferation and thus is involved in cardiomyocyte proliferations.[155] As shown in a mouse model, miR-208 is exclusively expressed in cardiomyocytes and consequently released during cardiomyocyte death in MI.[156] Two other miRs which were identified in some studies were miR-21 and miR-126. miR-21 is up-regulated in cardiomyocytes shortly after initiation of ischaemia. However, increased expression of miR-21 in fibroblasts enhances their proliferation,[157] suggesting a role of miR-21 in remodelling.[158] Levels of miR-126 were up-regulated in the non-infracted areas after induced MI in rat hearts. The effect of ischaemia reperfusion on miRs in the rat heart was evaluated by Tang *et al.*[159] The authors found that levels of miR-1, miR-126, and miR-208 were increased, while miR-21, miR-133, and miR-195 levels had decreased. Finally, we did not identify miR-29, although members of the miR-29 family were down-regulated in the region adjacent to MI areas in mice and humans.[160]

As stated earlier, miRs in plasma avoid degradation by being packaged in microvesicles (exosomes, microvesicles, and apoptotic bodies) or by being bound with proteins or HDLs.[161] In inflammatory microvesicles, miR-133 was among the most interesting miRs in association with metabolic and cardiovascular diseases, together with the let-7 family, miR-17/92 family, miR-21, miR-29, miR-126, miR-133, miR-146, and miR-155.[14] Other miRs were also identified as potential markers in plasma or serum, such as miR-126 predicted the occurrence of cardiovascular events in patients with stable CAD.[51] Independent study revealed a significant reduction of miR-126 expression in circulating microvesicles in patients with stable CAD with and without diabetes mellitus.[162] A group of miRs, including miR-21 and miR-126, were up-regulated in microvesicles isolated from the plasma of UA patients compared with controls.[11] In addition, miRs control the expression of most of the genes associated with HDL metabolism, including the ATP transporters, ABCA1, SRB1, etc. These findings suggest that miRs regulate HDL biogenesis, cellular cholesterol efflux, and HDL cholesterol uptake in the liver, thereby controlling all of the steps of reverse cholesterol transport, likely to be associated with cardiovascular disease.[163] For example, expression of ABCA1, associated with HDL, is highly regulated at the post-transcriptional level by multiple miRs, including miR-145**.** miRs also regulate the expression of LXR (liver X receptors), thereby controlling the transcriptional activation of ABCA1. LXR is directly targeted by miR-1 together with miR-206, miR-613, and miR-155.[164] In addition, HDL can transport miRs and deliver them to the receiving cells influencing their gene expression. Hence, it has been suggested that future studies should include data on miR expression in HDL particles or microvesicles. However, requirement of a high volume of plasma to isolate HDL and/or microvesicles and the current time consuming and expensive extraction procedures render such studies very unlikely, at least in the near future. Nevertheless, as seen in the studies included in this review, little or no information about presence in solution or package in HDL or microparticles was disclosed. Therefore, these data warrant further investigation of the potential of microvesicles as putative biomarkers and as novel carriers for the cell-specific transfer of miRs and other therapeutic agents.

4.6 Prognostic research

Further research is required and should concentrate on determining the prognostic value of miR deregulations before the onset of clinical cardiovascular symptoms. All studies in this review, except one, determined the association of miRs with the cardiovascular disease state at the time of blood sampling, and because of lack of follow-up of the patients, did not determine prognostic value of miRs to identify new events in addition to other risk factors. In order to do that, large-scale studies with longer patient follow-up periods are needed. This is where blood samples collected in biobanks could be useful as they represent both healthy and diseased populations. As blood samples in biobanks were collected with no particular goal initially, a majority of them are not suitable for detection of miRs other than in plasma or serum.

In conclusion, we reviewed the deregulated circulating miRs which may be useful for future miR research using blood samples from biobanks worldwide. We applied a systematic review approach to identify miRs which are more likely to be associated with CVD. Finally, we identified major limitations in current miR research and provided an overview of several aspects researchers should consider to prepare the highest-quality future studies and publications possible. Certainly, large-scale and standardized (references, adjustment for established cardiovascular and metabolic factors and medications) follow-up studies are needed to establish the prognostic value of miRs. Summarizing the most promising miRs, also by linking them to target genes implied in the development of CVD, could also be helpful in designing these future studies.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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